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DANIELE STÉFANIE SARA LOPES LERA NONOSE

Diferentes parâmetros *in vitro* e *in vivo* de três espécies de *Leishmania (Viannia)* no desenvolvimento da leishmaniose cutânea

Maringá
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências da Saúde, Área de concentração: Doenças Infecciosas e Parasitárias.

Orientadora: Prof.ª Dr.ª Maria Valdrinez Campana Lonardoni.
Co-orientadora: Prof.ª Dr.ª Izabel Galhardo Demarchi.

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Universidade Estadual de Maringá

Prof.ª Dr.ª Sandra Mara Alessi Aristides
Universidade Estadual de Maringá

Aprovada em:
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DEDICATÓRIA

Dedico este trabalho a DEUS, à minha família e aos meus amigos do laboratório de Imunologia Clínica da Universidade Estadual de Maringá

EPÍGRAFE

"(...) concedam ânimo ao vosso coração e vos fortaleçam para fazerem sempre o bem, tanto em atos como em palavras (...)."

2 Tessalonicenses 2:17

Diferentes parâmetros *in vitro* e *in vivo* de três espécies de *Leishmania (Viannia)* no desenvolvimento da leishmaniose cutânea

RESUMO

A leishmaniose cutânea (LC) pode ser causada pela *Leishmania (Viannia) braziliensis* [L(V)b], *L. (V.) lainsoni* [L(V)l] e *L. (V.) naiffi* [L(V)n], sendo a primeiro espécie também responsável pela forma mucosa e diferentes complexidades de LC e as outras duas por manifestações clínicas menos severas. Essas diferenças estão associadas à resposta imune do hospedeiro e a características intrínsecas do parasita. No entanto, as características parasitárias que contribuem para essas diferenças clínicas não são totalmente compreendidas, especialmente em termos de L(V)l e L(V)n. Este estudo teve como objetivo investigar aspectos *in vitro* e *in vivo* dessas três espécies, sendo a L(V)b representada por três diferentes cepas isoladas de pacientes com diferentes quadros de LC, que poderiam explicar essa diferentes manifestações clínicas da LC. Essas espécies apresentaram diferenças nos comprimentos de corpo, na forma, no perfil de peptidase extraídas da membrana de formas promastigotas e segregadas pelo hospedeiro, e índices de infecção. L(V)l teve o maior comprimento de corpo, forma fusiforme, sete bandas de peptidases relacionadas ao parasita e o menor índice de infecção. Por outro lado, L(V)n teve o menor comprimento de corpo, forma oval e quatro bandas de peptidases relacionadas ao parasita; apesar do padrão de bandas de enzimas secretadas pelo hospedeiro ter sido o mesmo entre todas as espécies/cepas, a banda de 72 KDa apresentou sua expressão diminuída apenas nesta espécie. Por fim, L(V)b teve um comprimento do corpo intermediário, forma fusiforme e três bandas de peptidases relacionadas ao parasita. As três cepas de L(V)b se diferenciaram apenas na expressão de peptidase relacionadas ao parasita, sendo duas das três bandas apresentadas por L(V)b menos expressas na cepa isolada de um paciente que apresentou uma resolução satisfatória da LC. Nenhuma das espécies/cepas estimulou a produção de derivados de nitrito em macrófagos. Na infecção *in vivo*, L(V)b foi a espécie mais virulenta com aumento significativo da lesão ($p = 0,001$) seguida de ulceração e uma carga parasitária constante de no linfonodo e no baço. A L(V)l teve uma virulência intermediária, com um aumento acentuado na lesão até a segunda semana após a infecção, seguida de uma estabilização no crescimento sem ulceração ($p = 0,001$), que no final de 105 dias de infecção foi menor que o aumento da lesão causada por L(V)b ($p = 0,05$) e a carga do baço e dos linfonodos foram positivas apenas na segunda semana. A L(V)n não desenvolveu lesões e a carga parasitária no linfonodo foi positiva apenas na segunda semanas de infecção. O peso dos animais não foi alterado pela

infecção causada por nenhuma das espécies. Estes resultados *in vitro* e *in vivo* mostraram que essas espécies diferenças apresentam características distintas que poderiam contribuir para os seus diferentes desfechos da LC, sendo L(V)b a espécie mais virulenta seguida por L(V)l e L(V)n, respectivamente.

Palavras-chave: leishmaniose cutânea; leishmaniose do novo mundo; virulência; *Leishmania*; interações hospedeiro-parasita, metaloproteases.

Distinct behavior *in vitro* and *in vivo* of three species of *Leishmania (Viannia)*in the development of cutaneous leishmaniasis

ABSTRACT

The cutaneous leishmaniasis (CL) can be caused by *Leishmania (Viannia) braziliensis* [L(V)b], *L. (V.) lainsoni* [L(V)l] and *L. (V.) naiffi* [L(V)n], being the former also responsible for the mucosal form and different outcomes in CL and the other two for less complicated clinical manifestations. These different outcomes are associated with host immune response and intrinsic parasite characteristics. However the parasite characteristics that contribute to these are not fully understand, especially in terms of L(V)l and L(V)n. This study aimed to investigate *in vitro* and *in vivo* aspects of these three species, being L(V)b represented by three different strain from patients with different CL outcomes, that could provide supporting facts for the broad CL clinical manifestations. These species presented different body lengths, forms, peptidase activity profiles related to enzymes in the membrane of promastigote forms (*Leishmania* peptidases) and secreted by the host (host peptidases), and infection indexes. L(V)l had the largest fusiform body length, seven bands of *Leishmania* peptidases and the lowest infective index. Otherwise, L(V)n had the smallest oval body length and four bands of *Leishmania* peptidases; even though the profile of bands from host peptidases was the same in all species/ strains, the band of 72 kDa was only diminished in L(V)n. Finally, L(V)b had intermediate fusiform body length and three bands of *Leishmania* peptidases. The three strains of L(V)b were different only in the expression of *Leishmania* peptidase, two out of three bands expressed by L(V)b were lowest expressed in the strain isolated from a patient with a satisfactory resolution of CL. No one of the species/strains stimulated nitrite-derived production by macrophages. In the *in vivo* infection, L(V)b was the most virulent specie with significant highest lesion enlargement ($p=0.001$) with ulceration, and a constant parasite load in lymph node and spleen. The L(V)l had an intermediate virulence, with a sharp increase in lesion sizes until the second week postinfection, followed by a stabilization without ulceration ($p=0.001$), that at the end of 105 days of infection was smaller than the L(V)b lesion ($p=0.05$), and the spleen and lymph node loads were just positive at the second week. The L(V)n did not develop lesions and the parasite load in lymph node was just positive two weeks after infection. The weight of animals were not affected by the infection with any species. These *in vitro* and *in vivo* results showed that these species had distinct characteristics that could contribute to their

different outcomes in CL, being the L(V)b the most virulent strain followed by L(V)l and L(v)n, respectively.

Keywords**:** cutaneous leishmaniasis; new world leishmaniasis; virulence; *Leishmania*; host-parasite interactions, metalloproteases.

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CAPÍTULO I

LEISHMANIOSES

Leishmanioses são zoonoses parasitárias de ampla distribuição que são socialmente e clinicamente negligenciadas. Profissionais de saúde, mesmo em países endêmicos, encontram dificuldades na identificação e manejo da doença e a população dessas áreas muitas vezes não então cientes de sua existência. A leishmaniose apresenta ocorrência habitual em 97 dos 200 países que se reportam a World Health Organization (WHO), com mais de 1 bilhão de pessoas vivendo nestas áreas que se apresentam em risco de contrair esta enfermidade (WHO, 2017a). De acordo com suas manifestações clínicas as leishmanioses podem ser categorizadas em três: visceral, cutânea e mucosa (BRASIL, 2017; WHO, 2017c), com mais de 616 milhões pessoas em situação eminente de contrair a forma visceral e mais de 431 milhões a forma cutânea (WHO, 2017a). Em 2016 forma registrados 5.076 casos no Brasil, sendo 212 na região sul e desses aproximadamente 95% dos casos aconteceram no Paraná (SISAN, 2017).

A forma visceral da leishmaniose também é chamada de Kala-azar, podendo ser caracterizada por inchaço do fígado e baço que pode levar a um quadro grave de anemia, seguida de uma drástica perda de peso com episódios de febres irregulares (Figure 1-A). Para países em desenvolvimento está forma tem alta taxa de mortalidade quando não tratada, sendo de 100% em crianças menores de 2 anos (BRASIL, 2017; WHO, 2017c). .

Apesar da leishmaniose cutânea não ter uma taxa de mortalidade proeminente como a da visceral, ela pode incapacitar gravemente os pacientes. A forma cutânea da doença é a mais comum, caracterizada por úlceras que aparecem normalmente nas partes expostas do corpo como pernas, braços e rosto (Figure 1-B). O número de úlceras por paciente pode variar drasticamente de 1 a 200, e quando desaparecem deixam cicatrizes inevitáveis que determinaram a extensão do dano causado ao paciente, que não incomum acabam prejudicando a vida socioeconômica (BRASIL, 2017; WHO, 2017c).

A forma mucosa é caracteriza por lesões destrutivas normalmente na mucosa nasofaringea (Figure 1-C). A forma clássica da leishmaniose mucosa é secundária à forma cutânea. No Brasil, das manifestações dermatológicas da leishmaniose notificada de 3- 6% são mucosa, sendo que em algumas áreas endêmica a leishmaniose mucosa pode ultrapassar 25% (Brasil, 2017).

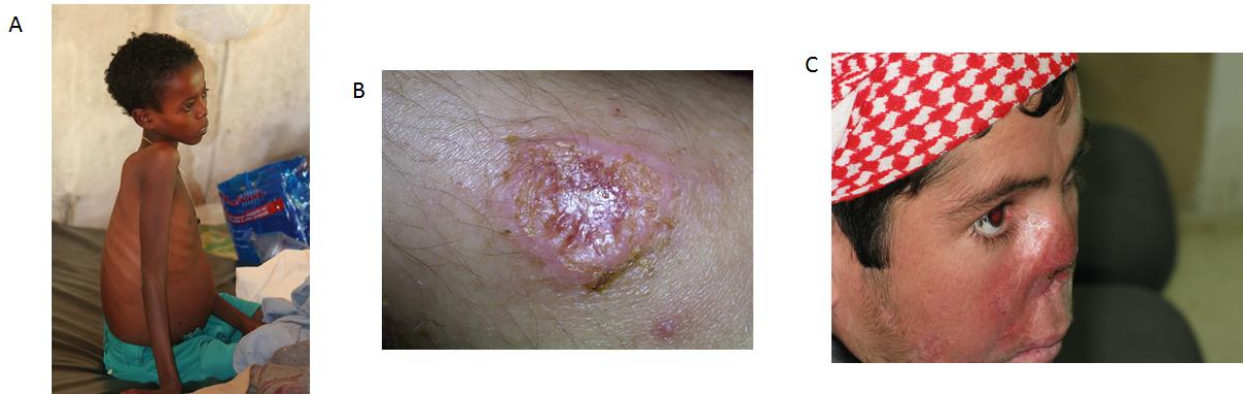

Figure 1- Manifestações clínicas da leishmaniose. A- Leishmaniose visceral. B- Leishmaniose cutâneas. C- Leishmaniose mucosa. Fone: WHO, 2017b.

HISTÓRICO DA LEISHMANIOSE TEGUMENTAR

O termo leishmaniose tegumentar (LT) é usado para englobar manifestações dermatológicas da leishmaniose, sendo associada a lesões cutâneas ulcerosas (leishmaniose cutânea) que algumas vezes causa comprometimento da mucosa do nariz e lábios (leishmaniose mucosa) (PESSOA e BARRETO, 1948). Uns dos primeiros relatos da LT ocorreram na Ásia Central nos primeiros 100 anos d.c, sendo chamada de "úlcera de Balkh" e/ou "úlcera de Delhi" (Camargo and Barcinski, 2003). Datadas entre o século IV e IX d.c, foram encontradas cerâmicas incas representativas de humanos com deformações orofaríngeas e cutâneas sugestivas de leishmaniose mucosa e cutânea respectivamente, também chamadas respectivamente no passado de espúndia (Figura 2-A) e uta (LAINSON e SHAW, 1989). Objetos cerâmicos e múmias da Cultura Mochica (100 a.c e 800 d.c) também foram encontrados com alterações cutâneas sugestivas de LT (Santos and Coimbra Júnior, 1994).

No Brasil, o relato mais antigo da LT foi feito em "Antiguedad de la syphilis en el Peru" de 1908 por Júlio César Tello citado no "Pastoral Religioso Político Geographico" de 1827. Nestes, há relatos de pessoas com feridas nos braços e nas pernas decorrentes de picadas de insetos que evoluíram para uma forma destrutiva das mucosas nasais e orais (Paraguassu-Chaves, 2001). No século XIX houve três irrefutáveis provas da LT no Brasil. O primeiro são estátuas de 1882-1884 com características indiscutíveis de LT encontradas no museu da Faculdade de Medicina do Rio de Janeiro. O segundo foi o caso de um italiano reportado por Brenda em 1884 que adquiriu LT no Brasil e retornou ao seu país de origem (Figura 2-B). O terceiro é uma ilustração de leishmaniose mucosa com 27 anos de evolução feita pela Sociedade Brasileira de Medicina em 1912 (Figura 2-C) (Furtado and Do Vale, 2005). Em 1895 foi

identificado o botão endêmico dos países quentes, chamando “Botão de Biskra” ou “Botão da Bahia” por Moreira (FURUSAWA e BORGES, 2014; NEVES et al., 2011).

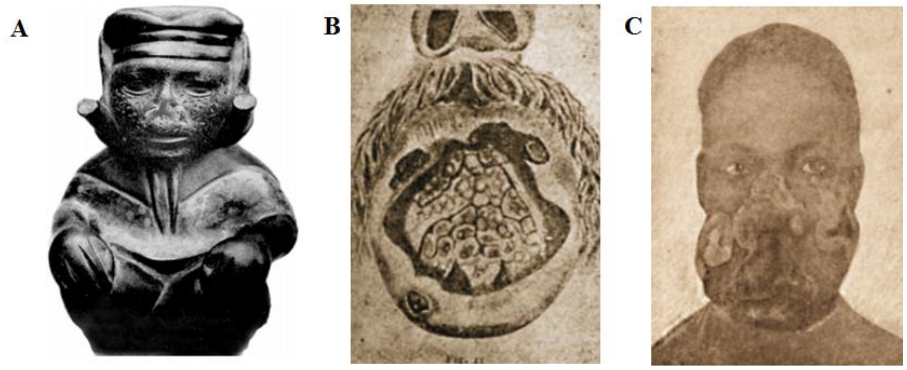

Figura 2- Marcos históricos da leishmaniose tegumentar. A- Huaco mochica exibindo mutilação do nariz e lábio superior sugestivas de espúndia (leishmaniose mucosa); fonte: ALTAMIRANO-ENCISO et al., 2003. B- Ilustração representativa da LT em um paciente italiano publicada por Breda (1884); fonte: FURTADO e DO VALE, 2005. C- Ilustração de um caso de LT publicado pela Sociedade Brasileira de Dermatologia em 1912; fonte: FURTADO e DO VALE, 2005.

A identificação do parasito nas lesões cutâneas e nasobucofaríngea só foi feita por Adolpho Lindengerg em 1909, em trabalhadores da construção de rodovias no interior de São Paulo. Dois anos depois (1911), Splendore diagnosticou a forma mucosa da doença e Gaspar Vianna identificou um parasito diferente da *Leishmania tropica*, responsável por causar a LT no velho mundo, chamado de *L. (Viannia) braziliensis*, sendo que a partir deste momento foi conhecido como único agente etiológico da LT no Brasil devido às limitações tecnológicas para a identificação dos parasitos. No ano seguinte, na 7° Congresso Brasileiro de Medicina e Cirurgia, Gaspar Vianna relatou os primeiros casos de LT tratados com o tártaro emético, antimonial trivalente. Em 1922, Aragão demonstrou pela primeira vez a transmissão da LT por flebotomíneos e em 1958 foi encontrado os reservatórios, roedores silvestres, da leishmaniose em florestas do Rio de Janeiro (Furusawa and Borges, 2014).

Após 1960, os parasitas passaram a ser classificados com base no comportamento em meios de cultura, em animais de experimentação e vetores, e não exclusivamente na manifestação clínica e evolução da doença, pois a microscopia óptica não permitia a distinção morfológica dos parasitos (Furtado, 1994; Furtado and Do Vale, 2005). Posteriormente, Lainson e Shaw dividiram o gênero *Leishmania* em dois complexos: mexicana e braziliensis (LAINSON e SHAW, 1972). Posteriormente em 1988, uma nova classificação das *Leishmania* foi proposta, incluindo o subgênero *Viannia* e *Leishmania*, elevando *Leishmania* do nível de subespécies para o de espécie (LAINSON e SHAW, 1988). Com o desenvolvimento das

técnicas imunológicas, bioquímicas e genéticas para a classificação dos parasitas sete novas espécies responsáveis pela LT humana no Brasil foram identificadas: *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) shawi*, *L. (V.) lainsoni*, *L. (V.) naiffi*, *L. (V.) lindenberg* e *L.(L.) amazonensis* (BRASIL, 2017; WHO, 2017b).

A partir de então, a LT tem sido relatada em várias cidades e em todos os estados do país e juntamente com isso houve mudanças no ciclo de transmissão. A princípio a LT era uma zoonose de animais silvestres que acometia o homem ao acaso. No entanto atualmente observa ciclos rurais e periurbanos com diversos agentes etiológicos e vetores com diferentes padrões de transmissão. Diante deste cenário o Ministério da Saúde propõe medidas de monitoramento, vigilância e de controle adequadas ao ciclo de transmissão, assim como ações voltadas para o diagnóstico oportuno e o tratamento adequado dos casos detectados (Brasil, 2017).

AGENTE ETIOLÓGICO E VETOR

As leishmanioses são doenças causadas por diferentes espécies de protozoários digenéticos. A classificação filogenética deste parasito é:

Reino: PROTISTA Haeckel, 1866
Sub-reino: PROTOZOA Goldfuss, 1817
Filo: SARCOMASTIGÓFORA Honigberg & Balamuth, 1963
Sub-filo: MASTIGOPHORA Desing, 1866
Classe: ZOOMASTIGOPHOREA Calkins, 1909
Ordem: KINETOPLASTIDA Honigberg, 1963, emend Vickerman, 1976
Sub-ordem: TRYPANOSOMATINA Kent, 1880
Família: TRYPANOSSOMATIDAE Doflein, 1901, emend. Globben, 1905
Gênero: *Leishmania* Ross, 1903
(GONTIJO e DE CARVALHO, 2003; LAINSON, 2010)

Uma forma flagelar chamada de promastigota encontrada no hospedeiro invertebrado mais precisamente no tubo digestivo (Figura 3-A), e outra aflagelada ou amastigota que é a forma intracelular obrigatória observada nos tecidos dos hospedeiros vertebrados principalmente nos vacúolos de macrófagos (Figura 3-B). A forma clínica da leishmaniose desenvolvida é dependente da interação do hospedeiro com o parasito, assim como da espécie infectante (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010; BRASIL, 2017).

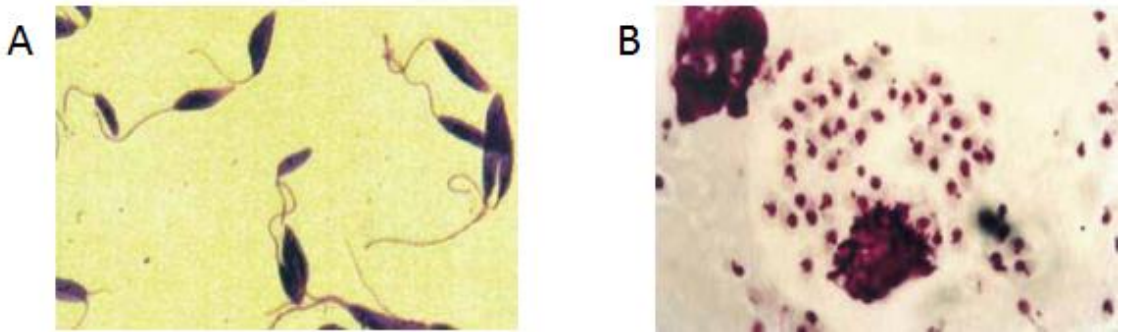

Figura-3: Formas do protozoário digenético *Leishmania*. A- Forma flagelar ou promastigota. B- Forma aflagelada ou amastigota.

O parasito *Leishmania* é digenético (heteroxênicos), ou seja, em seu ciclo de vida há a participação de mais de um hospedeiro, sendo um vertebrado (mamíferos, incluindo o homem) e o outro invertebrado que atua como vetor (insetos - flebotomíneos). A transmissão dos parasitos entre seus hospedeiros-reservatórios ocorre por meio do repasto sanguíneo de fêmeas infectadas. No homem, os parasitos infectam principalmente macrófagos transformando-se de promastigotas em amastigotas. Uma vez nos macrófagos, as amastigotas se multiplicam até romper a membrana celular e serem liberadas no tecido, reiniciando o ciclo (Brasil, 2017; Neves et al., 2011) (Figura 4).

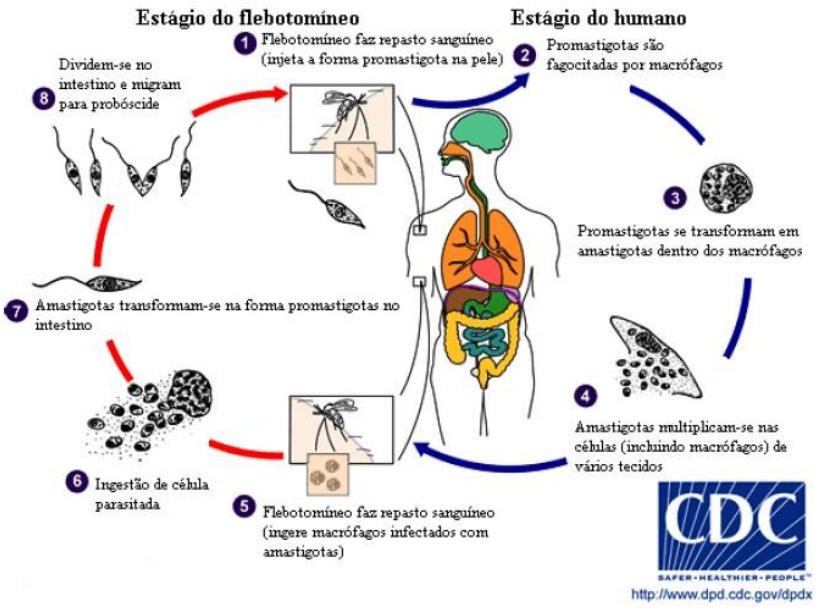

Figura 4- Ciclo de transmissão da leishmaniose. Fonte: Center for Disease Control and prevention (2010). Disponível em: https://goo.gl/cgrx69

No continente americano existem 12 espécies circulantes de *Leishmania* responsáveis pela leishmaniose humana e oito espécies restritas aos animais. Especificamente no Brasil, há sete espécies de *Leishmania* responsáveis pela LT, sendo uma do subgênero *Leishmania* e as outras do *Viannia* (Brasil, 2017):

L. (L.) amazonensis: agente da leishmaniose cutânea, assim como da forma difusa ou anérgica. Tem marsupiais e roedores como reservatórios e os seus vetores são *Lu. olmeca* e *Lu. flaviscutellata* (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010).

L. (V.) braziliensis: causadora da leishmaniose cutânea e mucosa é a mais predominante no Brasil. Está presente em todas as regiões endêmicas do país, estando relacionada à presença de animais domésticos. A transmissão desta ocorre por diferentes espécies de flebotomíneos: *Lu. witmani*, *Lu. wellcomei* e *Lu. intermedia*, entre outros (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010).

L. (V.) guyanensis: responsável pela forma cutânea, estando associada à regiões norte do Rio Amazonas com colonização recente. Os reservatórios são marsupiais e desdentados, sendo transmitido pelo *Lu. whitmani*, *Lu. anduzei* e *Lu. umbratilis* (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010).

L. (V.) lainsoni: restrita à Amazônia, causa a forma cutânea da leishmaniose e tem como reservatório suspeito a paca. O único vetor transmissor desta espécie, até o momento, é o *Lu. Ubiquitalis* (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010).

L. (V.) naiffi: assim como a *L. (V.) lainsoni* está circulando na Amazônia, incluindo os estados do Pará e Amazonas. Esta espécie é responsável por desenvolver LT normalmente de evolução benigna com diversos relatos de auto cura. O seu reservatório natural é o tatu e como vetores temos: *Lu. squamiventris*, *Lu. paraensis* e *Lu. Ayrozai* (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010).

L. (V.) lindenberg: foi identificada apenas em áreas desmatadas em Belém e Pará. Até o presente momento, esta espécie causa apenas leishmaniose cutânea sem relatos de mucosa e o único reservatório reportado é o homem, no entanto, há suspeita que os reservatórios silvestres sejam terrestres. Um grande candidato como seu vetor é *Lu. antunesi*, visto que não há vetores conhecidos (LAINSON, 2010).

L. (V.) shawi: no Pará e no Amazonas é responsável por casos esporádicos de LT. Os reservatórios são diversos, incluindo macacos, preguiças e procionídeos. O vetor é o *Lu. whitmani* (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010).

TRATAMENTO PARA LEISHMANIOSE TEGUMENTAR

O tratamento disponível para LT é composto por antimonial pentavalente, anfotericina B, pentamidina e pentoxifilina. O tratamento de escolha deve se basear na forma clínica da

leishmaniose, se respaldando em diagnóstico laboratorial e peculiaridades de cada situação (Brasil, 2017).

Existem duas formulações comerciais de antimoniais pentavalentes: o estibogluconato de sódio não comercializado no Brasil e o antimoniato de meglumina. A Organização Mundial da Saúde indica que a dose a ser administrada no paciente seja calculada em miligramas de antimônio pentavalente (Sb^{+5}) por quilograma de peso corporal por dia (mg Sb^{+5}/kg/dia). Este medicamento pode ser usado no tratamento de todas as formas clínicas da LT pelas vias intramuscular, intravenosa e intralesional. Os efeitos colaterais mais graves são toxicidade hepática, pancreática e cardíaca. Sua administração não é recomendada em gestantes (Brasil, 2017).

A anfotericina B, no Brasil, apresenta as seguintes formulações: anfotericina B lipossomal e desoxicolato de anfotericina B. Este medicamento pertence à classe de antibióticos, que se mostrou 400x mais potente que Sb^{+5} em hamsters infectados com *L. donovani*. Ambas formulações são administradas por infusão intravenosa lenta e podem gerar nefrotoxicidade, não sendo recomendadas para pacientes com insuficiência renal, apesar da toxicidade por anfotericina B lipossomal ser duas vezes menor em comparação como a outra (Brasil, 2017).

As diamidinas aromáticas representadas pelas pentamidinas são usadas em áreas endêmicas dos continentes americano para o tratamento da LT. A forma comercializada é o isetionato (Di-B-Hidroxietano Sulfonato) de pentamidina e é administrado por via endovenosa. Um dos efeitos colaterais deste medicamento é a toxicidade para células pancreáticas que pode desencadear diabetes, além de leucopenia, trombocitopenia, pancreatite, arritmias cardíacas, hipocalcemia, taquicardia ventricular, insuficiência renal aguda, e choque anafilático. Não é recomendado para gestantes, diabéticos e menores de um ano (Brasil, 2017).

A pentoxifilina tem sido usada como imunomodulador no tratamento da LT. A sua indicação é em associação com Sb^{+5}. Este é um vasodilatador periférico com propriedade hemorreológicas. A forma farmacêutica é um comprimido revestido com liberação prolongada e miligramagem de 400 mg. A sua associação em estudos duplo cego demonstrou diminuição da toxicidade do antimoniato por aumentar a taxa de cura além de acelerar este processo o que evita o uso de um segundo ciclo de antimoniato. Em alta concentração, a pentoxifilina comumente causa distúrbios gastrintestinais (sensação de pressão gástrica, plenitude, náusea, vômito ou diarreia) e flush (rubor facial com sensação de calor) (Brasil, 2017).

METALOPROTEASES

As metaloproteases são um conjunto de enzimas importantes para a proliferação celular, diferenciação, remodelamento extracelular, vascularização e migração celular em processos biológicos normais do desenvolvimento e patológicos (Chang and Werb, 2001). Estas peptidases podem ser produzidas por parasitas e hospedeiros, estando relacionadas com diferentes estágios da leishmaniose e podem influenciar diretamente o curso das doenças.

METALOPROTEASES DE MEMBRANA DA FORMA PROSMASTIGOTA DE *Leishmania* sp.

As peptidases encontradas na superfície de formas promastigotas estão relacionadas a vários processos de desenvolvimento e proliferação, contribuindo para a interação com o hospedeiro (vertebrados ou invertebrados) e evasão da resposta imune pelos parasitos (ISNARD et al., 2012; MCKERROW et al., 1993; MOTTRAM et al., 1998; VERMELHO et al., 2007; YAO et al., 2003). Uma comparação do perfil enzimático de isolados clínicos de pacientes com diferentes manifestações clínicas da LT, demonstrou diferentes perfis enzimáticos com peptidases que variaram de 50-125 kDa (CUERVO et al., 2005, 2008). Um perfil enzimático diferente também foi encontrado entre cepas virulentas e não virulentas *de L. (V.) brasiliensis*, enquanto que a estirpe virulenta expressava quatro bandas de pepitidases e a não virulenta apenas uma (LIMA et al., 2009).

A glicoproteína 63 (GP63) é a metaloprotease de membrana que se destaca das demais, estando fortemente associada a virulência do parasito *Leishmania* sp. (OLIVEIRA et al., 2013). Esta é uma enzima multifuncional, estando relacionada com a inativação de componentes do complemento (ISNARD et al., 2012), degradação da matriz extracelular contribuindo para a migração celular (ISNARD et al., 2012; YAO et al., 2003), inibição das células NK e ativação da tirosino fosfatase do hospedeiro (GOMEZ et al., 2009; SHIO e OLIVIER, 2010).

METALOPROTEASES SECRETADAS PELO HOSPEDEIRO VERTEBRADO EM RESPOSTA A INFECÇÃO POR *Leishmania* sp.

No controle da infecção por *Leishmania* se destacam os macrófagos e os monócitos, pois são as principais células infectadas e participam do processo de eliminação e disseminação do parasito (RIBEIRO-GOMES et al., 2014; SCOTT e NOVAIS, 2016). Recentemente, constatou-se que monócitos circulantes e macrófagos ativados podem produzir MMPs em

pacientes com leishmaniose cutânea (KOCH et al., 2010; ROSSOL et al., 2012; CAMPOS et al., 2014).

As MMPs na resposta inflamatória primária regulam a permeabilidade microvascular e recrutam leucócitos (CHEN et al., 2013). Na patogênese da LT, a MMP-9 e MMP-2 têm se destacado. Ambas MMPs tem como principal substrato o colágeno tipo IV, componente abundante na membrana basal cutânea (VEIDAL et al., 2011; JABŁOŃSKA-TRYPUĆ et al., 2016). Em pacientes com leishmaniose mucosa, mais especificamente em polimorfonucleares isolados do sangue periférico, a ativação de MMP-9 pode estar associada com a disseminação do parasito *L. (V.) brasiliensis* (MARETTI-MIRA et al., 2011). Uma alta atividade de MMP-9 também foi relacionada com leishmaniose dermal pós-calazar (ISLAM et al., 2013). Corroborando este resultado, uma alta atividade de MMP-9 associada à baixa produção de MMP-2 foi observada na progressão da fase pré-ucerativa para a ulcerativa na leishmaniose cutânea (CAMPOS et al., 2014). A partir destes resultados é possível inferir que a MMP-9 está envolvida em uma evolução mais severa da doença enquanto que a MMP-2 é um bom prognóstico. Com relação a resposta terapêutica, a MMP-2 também se mostrou relacionada a uma boa evolução da doença; pacientes que evoluíram para a cura após tratamento com Sb^{+5} em comparação com os que não alcançaram o mesmo desfecho e tiveram uma produção aumentada de MMP-2 (MARETTI-MIRA. et al., 2010).

JUSTIFICATIVA

A leishmaniose é uma doença com diferentes manifestações (World Health Organization, 2017b) e estas estão relacionadas com a espécie de *Leishmania* responsável pela infecção. *L. (V.) naiffi* e *L. (V.) lainsoni* são responsáveis apenas pela forma cutânea da leishmaniose, sendo que a primeira espécie tem vasto relatos de cura espontânea na literatura; por outro lado a *L. (V.) braziliensis* é responsável pela forma cutânea e mucosa (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010; BRASIL, 2017). Outra diferença interessante entre estas espécies é a taxa de incidência de leishmaniose humana, sendo no Brasil a leishmaniose causada por *L. (V.) braziliensis* a mais frequente (Brasil, 2017). Uma explicação para esta diferença estaria ligada aos vetores de cada uma, pois a *L. (V.) braziliensis* apresenta um maior número de espécies transmissoras (GONTIJO e DE CARVALHO, 2003; LAINSON, 2010). No entanto, os fatores intrínsecos de cada espécie que contribuem para estas distintas manifestações não são elucidados. Além das diferenças entre espécies que determinam diferentes manifestações da leishmaniose, estudos têm demonstrado a existência variações intra

espécie que associada à características imunes do hospedeiro também contribuem para diferentes desfechos (Oliveira et al., 2013).

Com base nesse cenário, um estudo que investigue características intra e inter espécies de *Leishmania* spp. é de extrema relevância. Pois pode contribuir para a elucidação de fatores de prognóstico a partir da identificação de características que contribuam para permitir a ocorrência de uma leishmaniose mais benigna. Além disso, a constatação de características específicas do parasito que contribuam para uma evolução maligna da doença podem contribuir para estudos sobre novos alvos terapêuticos.

OBJETIVO

GERAL

Investigar diferentes aspectos *in vitro* e *in vivo* de três espécies de *Leishmania (Viannia)* spp., bem como de três cepas diferentes de *L. (V.) braziliensis* isoladas de pacientes com diferentes desfechos na leishmaniose, buscando fatores que possam explicar as diferentes manifestações clínicas de leishmaniose ligada a cada espécie/cepa.

ESPECÍFICO

Estudar aspectos morfológicos das espécies: *L. (V.) lainsoni*, *L. (V.) naiffi* e *L. (V.) braziliensis*, sendo que a última espécie foi representada por três cepas: uma isolada de paciente que respondeu satisfatoriamente ao tratamento com Glucantime®, outra isolada de um paciente sem resposta ao tratamento e a última é uma cepa padrão do Laboratório de Imunologia Clínica da Universidade Estadual de Maringá.

Determinar a infectividade de cada espécie/cepa *in vitro.*

Avaliar diferenças quanto a estimulação de derivados de nitrito em resposta a infecção por *Leishmania* spp em macrófagos.

Investigar diferenças no perfil enzimático de membrana das espécies/cepas.

Investigar diferenças no perfil enzimático secretado por macrófagos em resposta à infecção causada por cada espécie/cepa.

Acompanhar a evolução e desfecho da leishmaniose causada por cada espécie em golden hamsters.

Quantificar a carga parasitária no linfonodo, baço e fígado na infecção por *Leishmania* spp. *in vivo.*

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CAPÍTULO II

Artigo: “DISTINCT BEHAVIOR *in vitro* AND *in vivo* OF THREE SPECIES OF *Leishmania (Viannia)* subgenus”

TITLE PAGE

TITLE: DISTINCT BEHAVIOR *in vitro* AND *in vivo* OF THREE SPECIES OF *Leishmania (Viannia)* subgenus

AUTHORS: Daniele Stéfanie Sara Lopes Lera-Nonose[1], Áquila Carolina Fernandes Herculano Ramos Milaré[1], Camila Alves Mota[1], Giovanna Chiqueto Duarte[2], Thaís Gomes Verzignassi Silveira[3], Izabel Galhardo Demarchi[3]; Maria Valdrinez Campana Lonardoni[3].

[1] Post-Graduate student at Health Sciences Graduate Program, Universidade Estadual de Maringá, Avenida Colombo, 5790, Jardim Universitário, CEP 87020-900, Maringá, Paraná, Brazil.

[2] Undergraduate student of medicine, Universidade Estadual de Maringá, Avenida Colombo, 5790, Jardim Universitário, CEP 87020-900, Maringá, Paraná, Brazil.

[3] Department of Clinical Analysis and Biomedicine, Universidade Estadual de Maringá, Avenida Colombo, 5790, Jardim Universitário, CEP 87020-900, Maringá, Paraná, Brazil.

CORRESPONDING AUTHOR: Daniele Stéfanie Sara Lopes Lera Nonose, Health Sciences Graduate Program, Universidade Estadual de Maringá, Avenida Colombo, 5790, Jardim Universitário, CEP 87020-900, Maringá, Paraná, Brazil, +55 (44) 3011-4878 /+55 (44) 3011-1376 and daniele.s.s.l.lera@gmail.com

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NOTE: supplementary data associated with this article

ABSTRACT

The cutaneous leishmaniasis (CL) can be caused by *Leishmania (Viannia) braziliensis* [L(V)b], *L. (V.) lainsoni* [L(V)l] and *L. (V.) naiffi* [L(V)n], being the former responsible for the mucosal form and different outcomes in CL and the other two for less complicated clinical manifestations. There are different outcomes associated with host immune response and intrinsic parasite characteristics, especially unclear for L(V)l and L(V)n. We investigated *in vitro* and *in vivo* aspects of these three species that could provide supporting facts for the different leishmaniasis outcomes. These species presented different body lengths, forms, peptidase activity profiles related to enzymes in the membrane of promastigote forms (*Leishmania* peptidases) and infection indexes. L(V)l had the largest fusiform body length, seven bands of *Leishmania* peptidases and the lowest infective index. Otherwise, L(V)n had the smallest oval body length and four bands of *Leishmania* peptidases. Finally, L(V)b had intermediate fusiform body length and three bands of *Leishmania* peptidases. In the *in vivo* infection, L(V)b was the most virulent strain with significant highest lesion enlargement (p=0.001) followed by ulceration, and constant L(V)b load in lymph node and spleen. The L(V)l had an intermediate virulence, with a sharp increase in lesion sizes until the second week postinfection, followed by a stabilization without ulceration (p=0.001) that at the end of 105 days of infection was smaller than the L(V)b lesion, and the spleen and lymph node load were just positive at the second week. The L(V)n did not develop lesions and the parasite load was just positive two weeks after infection. The *in vitro* and *in vivo* results showed that these species have difference in virulence and peptidase activity profile that could be related to their clinical manifestation in humans.

KEYWORDS: cutaneous leishmaniasis; new world leishmaniasis; virulence; *Leishmania*; host-parasite interactions; metalloproteases.

1-Introduction

Leishmaniasis is a set of zoonotic diseases with high complexity in terms of clinical manifestations and epidemiology with worldwide distribution affecting mainly developing countries,

the poorest, representing a large impact on their health economy (WHO, 2017a). The more frequent form is the cutaneous leishmaniasis (CL) (WHO, 2017b), with more than one million cases reported in the last five years, being 90 % of the cases in Bolívia, Brazil and Peru (WHO, 2017c). The main species for CL in the New World (the Western Hemisphere) are either in the *Leishmania (Viannia) braziliensis* species complex including also *L. (V.) guyanensis*, *L. (V.) panamensis* and *L. (V.) peruviana*; and *Leishmania (Leishmania) mexicana* species complex: *L. (L.) mexicana*, *L. (L.) amazonensis*, and *L. (L.) venezuelensis*. Reminding that the *L. (V.) braziliensis* are related with the mucosal leishmaniasis too, and the outcomes of leishmaniasis caused by this specie is wide (CDC, 2017). These divergent outcomes may be a result of individual host immune system and may be associated with intrinsic characteristics of the infecting strain (Oliveira et al., 2013). Other species from the subgenus *Viannia* also develop CL with better prognosis and lower incidence, such as *L. (V.) lainsoni* (Jennings et al., 2014; WHO, 2010) and *L. (V.) naiffi* (Figueira et al., 2014; WHO, 2010), causing unique ulcers and the latter specie is related to self-limiting infections (WHO, 2010).

Leishmania is transmitted to vertebrate hosts by the bite of sandflies/ phlebotomine. The sandflies responsible for leishmaniasis transmission belong to the genus *Lutzomyia*, and the species variating according to the transmitted *Leishmania* specie. The *L. (V.) lainsoni* is reported to be transmitted, until the moment, only by *Lu. ubiquitalisé*, a phlebotomine of low anthropophilic. In this same line, *L. (V.) naiffi* is mainly transmitted by *Lu. ayrozai*, another phlebotomine with low anthropophilic, although there are rare cases of its transmission by *Lu. paraensis* and *Lu. squamiventris*, which are highly anthropophilic. On the other hand, *L. (V.) braziliensis* has numerous sandfly species involved in its transmission such as *Lu. complexa*, *Lu. wellcomei*, *Lu. whitmani*, *Lu. migone*, *Lu. neiva* and *Lu. intermedia*, all of them highly anthropophilic (Gontijo and de Carvalho, 2003; Lainson, 2010). This difference in vectors could be the reason why *L. (V.) braziliensis* has the highest rate of infection in humans. However, these cannot fully explain those differences in infection rates among the species and some intrinsic factor of parasites must be associated with less or more virulence against human host.

Considering the different leishmaniasis outcomes related to parasite intrinsic characteristics, we investigated the different aspects *in vitro* and i*n vivo* of three species of *Leishmania (Viannia)* spp., as well as three different strains of *L. (V.) braziliensis* with distinct CL outcomes. Targeting supporting facts for different leishmaniasis clinical manifestations due to infecting specie/ strain. The relevance of this study relay in the fact that basic researches, especially in the parasite field and host-parasite interaction can contribute in the elucidation of new drug targets, and prognostic and diagnostic factors (Maretti-Mira et al., 2010; Vermelho et al., 2007). In our knowledge, this is the first study researching *L. (V.) naiffi* and *L. (V.) lainsoni* in animal models with described methodology.

2-Material and Methods

2.1-Parasites and culture growth conditions

This study used five strains of *Leishmania (Viannia)* spp., being three strains of *L. (V.) braziliensis* [L(V)b]: one isolated from a patient with complete regression of the lesion after the Glucantime® treatment MHOM/2003/2314 [L(V)b-M2314], another from a patient that did not have healing after the treatment MHOM/BR/2009/3476 [L(V)b-M3476] and the last one was MHOM/BR/1987/M11272 [L(V)b-M11272] (Fernandes et al., 2016), that is a well-known strain of L(V)b in the Laboratory of Clinical Immunology at the Universidade Estadual de Maringá (UEM) that was isolated from a case of CL; one strain of *L. (V.) lainsoni* MHOM/BR/8l/M6424 [L(V)l] isolated from a patient with CL and another of *L. (V.) naiffi* MDAS/BR/79/M5533 [L(V)n] that was isolated from an armadillo. All strains ($5x10^6$ parasites/mL) were kept by weekly seed at 25 ºC in 199 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% (V/V) inactivated fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA) and antibiotics (penicillin G 0.1 UI/mL, streptomycin 100 ng/mL, Sigma-Aldrich, Saint Louis, MO, USA).

2.2-Culture and growth conditions of J774A.1 macrophages

The macrophage-like cell line J774A.1 (ATCC: TIB67, *Banco de Células do Rio de Janeiro*, RJ, Brazil) were cultivated in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 20% (V/V) FBS and antibiotics (penicillin G 0.1 UI/mL, streptomycin 100 ng/mL) at 37 ºC with 5% of carbon dioxide (CO_2).

2.3-Morphologic assay

Promastigote forms in stationary phase were centrifuged at 2500 rpm for 10 min and washed twice with phosphate-buffered saline buffer pH 7.2. Following, they were fixed with 2% formalin, a drop of each strain was put in a slide and stained with Giemsa (Sigma-Aldrich, Saint Louis, MO, USA) and observe at EVOS FL Imaging System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 100x magnification. The body length was estimated by the measure of 2 promastigotes per field of at least 10 fields based on the scale provided by the microscopy (Rogers et al., 2002 modified).

2.4-Infectiveness *in vitro*

A suspension of $1x10^6$ J774A.1 macrophages per mL was distributed in a plate of 24 wells with glass coverslips, followed by 2 h of incubation at 37 ºC with 5% of CO_2. Following, seven parasites in stationary phase were added per macrophage and the plate reincubated for 4 h. At the end, all coverslips were washed and part of coverslips was stained with Panotico kit (Laborclin, Vargem Grande, Pinhais, Brazil) (time zero "0 h") and the other part was reincubated with RPMI-1640 medium for more 24 h to analyze the behavior of macrophage- *Leishmania* interaction. Following, the coverslips were stained with Panotico kit (time twenty-four hours "24 h") and the supernatants were stored for further analysis (nitrite-derived production, and zymographic and inhibition assay). In microscopy (Olympus, Shinjuku, Tokyo, Japan), 200 macrophages were counted, quantifying the infected (I) and uninfected ones, the number of parasites per macrophage was also assessed (P). The infection index was estimated by the expression [P x (I x 100/ 200)] (Cardoso et al., 2015 modified). The experiments were performed in triplicate.

2.5- Nitrite-derived production

The supernatants from infectiveness *in vitro* assay were also used to determine the production of nitrite-derived by the method described by Fernandes et al. (2016) modified. For this, the supernatant was incubated with Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% orthophosphoric acid) in the proportion of 1:1 at room temperature for 15 min. The absorbance was read at 550/620 nm in a spectrophotometer (VersaMax™ ELISA Microplate Reader, Molecular Device, Sunnyvale, California, USA), the concentration of nitrite-derived was based on a standard curve of sodium nitrite ($NaNO_2$). The experiments were performed in triplicate. Supernatant of macrophages stimulated with lipopolysaccharide (Sigma-Aldrich, Saint Louis, MO, USA) was used as a positive control of nitrite-derived production (Brunner et al., 2015).

2.6-Zymographic and inhibition assay

For zymographic assay, peptidases present in promastigote forms of parasites (*Leishmania* peptidases) and peptidases secreted by macrophages in response to *Leishmania* infection (host peptidases) were isolated. Peptidases present in stationary phase of parasites were obtained by disrupting $1x10^8$ promastigote forms in 100 μL of lysis buffer containing 4% of sodium dodecyl sulfate (SDS) in 0.1 M TRIS, pH 6.8, followed by three cycles of vortex for 1 min with breaks of 4 min in ice (Lima et al., 2009 modified). Host peptidases produced by infected and uninfected macrophages were obtained by concentrating 1.5 mL of the supernatant from the infectiveness *in vitro* assay with 0.75 mL of absolute ethanol (LabSinth, Diadema, SP, Brazil), followed by an incubation at -30 °C for 24 h. After that, this mixture was centrifuged at 4000 rpm for 15 min and the pellet resuspended in 0.5 M of TRIS, pH 6.8. The same procedure was made with RPMI-1640 medium as a control.

The proteins present in both type of extracts were measured by Qubit™ Protein Assay Kit (Invitrogen, Eugene, Oregon, USA). The extract of *Leishmania* peptidases (50 μg) and host

peptidases (1 μg) were mixed with sample buffer and applied to 10 and 8% polyacrylamide SDS-PAGE, both with 0.1% of gelatin (Sigma-Aldrich, Saint Louis, MO, USA), respectively. Sample buffer alone was also added into the gels as an internal control. After electrophoresis, the resulting gels were washed twice for 30 min under agitation at room temperature with 2.5% triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA). Protease activities were detected for both extracts by incubating the gels in the renaturation buffer (50 mM Tris, 75 mM NaCl and 2.5 mM $CaCl_2$) in pH 5.5 and 7.2, respectively, at 37^0C for 48 h. The gels were stained with 0.2% (w/v) Coomassie blue, 30% (v/v) methanol, 10% (v/v) acetic acid, and destained with 30% (v/v) methanol, 10% (v/v) acetic acid.

For inhibition assays, the metalloprotease inhibitor 1,10-phenanthroline (Sigma-Aldrich, Saint Louis, MO, USA) (10 mM for *Leishmania* peptidase and 5 mM for host peptidase) was added in the renaturation buffer. The weights of peptidases were measured based on a molecular weight standard (SeeBlue™ Prestained Protein Ladder, Invitrogen, Eugene, Oregon, USA). The experiments were performed in triplicate.

The gels were digitized by a scanner for the densitometric analysis. The peptidase activities were estimated based on the intensity of light bands by the Image J 1.46 software (National Institutes of Health, Bethesda, Maryland, USA). Initially, the image was transformed to 8-bits, and a rectangular involving the bands and the dark background were manually selected. The contrast of these elements was graphically demonstrated by inverted pics that were proportional to peptidase activities in the gel. For minimizing the error in the manual selection of area, this measure was made three times for each gel and at least two gels were used (Da Silva et al., 2014).

2.7-Golden hamsters

Adult male golden hamsters provided by the Central Animal House of UEM were used in this study. The animals were kept in Mini-isolators Ventilife (Alesco, Monte Mor, São Paulo, Brazil) that injected air directly into the cage with a slow continuous flow, ensuring the perfect air renew and the

inter pressure balance. The animals received *ad libitum* water and feeding with controlled ambient temperature (22 ^{0}C), humidity (70%), 12 h light dark cycles and in an ambient with physical barrier. The animals were used following the rules of 3R.

2.8-Euthanasia of golden hamsters

The animals were pre-anesthetized with isoflurane (anesthetic gas). After the anesthesia was confirmed, they were euthanized in the carbon dioxide (CO_2) chamber, as established by Resolution n^0 100, of May 11th, 2012 by the Brazilian Federal Council of Veterinary Medicine; Brazilian Guide to Good Practices in Animal Euthanasia: Recommended Concepts and Procedures (Portuguese: *Guia Brasileiro de Boas Práticas em Eutanásia em Animais: Conceitos e Procedimentos Recomendados*) (2013); Euthanasia Practice Guidelines of CONCEA (Portuguese: *Diretrizes da Prática de Eutanásia do CONCEA*) (2015); and the Commission of the European Communities Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes.

2.9-Infection of golden hamsters

For the infection of golden hamsters, the L(V)b-M2314 was chosen as a representative of L(V)b specie, because there were no major difference among the three studied strains of L(V)b, in other words, all L(V)b stain were similar in the *in vitro* experiments. The L(V)l and L(V)n were also used. Each strain had three groups and one control group for all of them, each group had five animals. Promastigote forms of *Leishmania (Viannia)* spp. in stationary phase ($5x10^6$ promastigotes/ 100 μL of PBS) were inoculated in the right hind paw (RP) of male golden hamster, and on the left hind paw (LP) only PBS was inoculated as a control. The animal's paw was measured by paquimeter and lesion enlargement was calculated (RP-LP). This parameter and the weight of animals were monitored weekly for 15 weeks. The right popliteal lymph node, spleen and liver were collected at 2nd, 8th, and

15th week postinfection. The samples were weighed and part of each organ was separated for parasite load analysis by limiting dilution and Real Time Polymerase Chain Reaction (qPCR).

2.10-Parasite load by limiting dilution

A known weight of right popliteal lymph node, spleen or liver was triturated and diluted 1/100 in 199 medium. In a plate of 96 wells, 25 μL of this suspension was added at the first well with more 75 μL of supplemented 199 medium, from this well a serial dilution ½ was carried on. All organs were diluted in duplicate, and the plates were read in microscopy after seven days. The title of the last well with parasite was analyzed and the result was expressed in log of the arithmetic mean of the last positive title divided by the weight of the organ (Cardoso et al., 2015, modified).

2.11-Parasite load by qPCR

2.11.1- Tissue preparation and DNA extraction

To evaluate the hematogenic dissemination of parasites in the animal model, the parasite load in spleen was evaluated by qPCR (Wakimoto, 2009), more sensitive than limiting dilution technic. For this, a known weight of spleen was triturated, transferred to an eppendorf with 300 μL of ACD (Acid Citrate Dextrose). The DNA equivalent to 10 mg of tissue was extracted using Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations.

2.11.2- qPCR procedure

The parasite load was assessed by absolute quantification method. For this, a serial dilution curve (DNA equivalents to $5x10^{4}$ to $5x10^{-3}$ *Leishmania*) was constructed using *L (V.) braziliensis* MHOM/BR/75/M2903 (strain of *Leishmania* with identified genome) (S1-A). In parallel, a standard curve to detect the GAPDH gene (constitutive gene of golden hamster) was constructed with the DNA extracted from 0.4 mg of spleen tissue, serially diluted until the DNA corresponding to $4x10^{-6}$ mg (S1-B). To detect *Leishmania* parasites, the minicircle kDNA gene of *Leishmania (Viannia)* sp. was

amplified with the primers MP3H (5'-GAA CGG GGT TTC TGT ATG C-3') and MP1L (5'-TAC TCC CCG ACA TGC CTC TG-3') (Lopez et al., 1993). The constitutive gene GAPDH was amplified with the primers Fw 5′-GGT TGC CAA ACC TTA TCA GAA ATG-3′ and Rv 5′-TTC ACC TGT TCC ACA GCC TTG-3′ (Ribeiro-Romão et al., 2014).The reaction mixture was carried out in 10 µL final volume, containing 500 nM MP3H/750 nM MP1L or 100 nM from forward and reverse primer for GAPDH gene (Ribeiro-Romão et al., 2014), 1X Sybr® Green Select Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1 µL of DNA sample. The reaction was run in a StepOne PlusTM Real-Time PCR Systems (Applied Biosystems, CA, USA) with an initial heating at 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 sec and 68 ºC for 1 min. By the end of PCR amplification, a melting curve was generated, being one cycle at 95 ºC for 15 sec, followed by 60 ºC for 1 min and continuous heating at 0.3 ºC/sec to 95 ºC. The threshold was automatically determined by StepOne™ Software v2.1. The melting curves were used to check the primers specificity (S1-C and D). First, the kDNA and GAPDH standard curves were run to verify the amplification efficiency of the chosen primers and then, both were run together the samples (S1-E and F). The results were expressed as the number of parasites per mg of tissue.

2.12- Statistical analysis

All results were plotted at Excel (Microsoft Corporation, Albuquerque, New Mexico, USA) and the results were expressed as means ± standard deviations. For comparison between two groups with normal distribution by the Shapiro-Wilk test the t-student test was used and when the data did not followed normal distribution the Mann-Whitney test was applied. In the analysis that included more than two groups, the analysis of variance by ANOVA followed by the Tukey post hoc test was used, since the data followed the criteria of homogeneity of variance and residuals analysis. In the cases that data did not meet these criteria, Kruskal-Wallis followed by the Mann-Whitney pairwise test was used. All tests were performed at the Past 3.X software (University of Oslo, Oslo, Norway). Statistical differences were considered at $p = 0.05$.

2.13-Ethical aspects

The project was approved by the Ethical Conduct Committee on animal use of the UEM under the protocol number 3062060916.

3-Results

3.1-Morphologic assay

In general, the three species of *Leishmania (Viannia*) spp. showed different promastigote forms. There was no difference related to morphology among the three strains of L(V)b, the body lengths of L(V)b strains ranged from 7.55 to 8.50 μm with body width of approximately 1μm and the flagellum seemed to be bigger than the body length, without significant statistical difference among their body length. The L(V)n was the smallest, with a body length of 4.82±0.62 μm, body width variable and the flagellum seemed to be smaller than the body length. The L(V)l was the largest specie with a body length of 11.71±1.95 μm ($p=0.001$), body width variable and the flagellum size variable. Differences in the promastigote forms were also found, being the L(V)n oval and the others fusiform (Figure 1).

3.2-Infectiveness *in vitro*

The infection indexes on macrophages of all *Leishmania (Viannia)* spp. at 0 h were statically equal ($p>0.05$), being them 188±29, 204±31, 180±19, 185.42±31 and 200.67±26 for L(V)b-M2314, L(V)b-M3476, L(V)b-M11272, L(V)l and L(V)n, respectively. Otherwise, the infection index after 24 h was lowest in L(V)l with 124±24 ($p=0.001$). The others *Leishmania (Viannia)* spp. had infection indexes of 213±28, 208±35, 181±33 and 183±28 for L(V)b-M2314, L(V)b-M3476, L(V)b-M11272 and L(V)n, respectively. The comparison of the infection indexes at 0h and 24h showed that it was significantly reduced in the L(V)l ($p=0.001$) (Figure 2).

The nitrite-derived production in the positive control with LPS was of 41.37±8.02 μM. In all studied strains and species, nitrite-derived production was below 2.07 μM, while the basal production of these compounds by macrophages alone was of 1.21±1.05 μM. These results indicate that nitrite-derived production was not stimulated in any infection by *Leishmania (Viannia)* spp. (S2).

3.3-Peptidase activity profile

The analysis of *Leishmania* peptidases showed the difference among the species. The L(V)l presented the largest number of bands (seven), being them 141, 73, 67, 60, 57, 55 and 51 kDa and the band that had the highest expression was the 55 kDa. The L(V)n had four bands and two of those were the 57 and 55 kDa, which were also present in the peptidase activity profile of L(V)l, the other two bands were 78 and 62 kDa, with highest expression of the lowest molecular weight. There was no difference in *Leishmania* peptidase profiles among the three strains of L(V)b that had three bands of 92, 78 and 62 kDa, being the lightest band with major expression. Highlighting that these last two were present in the peptidase activity profile of L(V)n and L(V)b (Figure 3A). Although there were no different bands expressed in the L(V)b strains, when the levels of expression of these bands were analyzed, differences were found. The lightest band was equally expressed by the strains and the other two (92 and 78 kDa) were diminished in L(V)b-M2314 ($p=0.004$ and $p=0.003$) (Figure 3B). In presence of 1-10 phenanthroline the majority of bands were completely inhibited, except the band of 62 kDa in L(V)b strains and the band of 52 kDa in L(V)l that were partially inhibited (S3-A).

The host proteolytic activity was not different among uninfected macrophages and infected ones, presenting six bands of 130, 98, 72, 68, 37 and 34 kDa (Figure 3C). Comparing the expression of the band with highest expression (72 kDa), it was down regulated in the infection caused by L(V)n in comparison to the uninfected macrophages and infected ones by the three strains of L(V)b ($p=.0.005$) (Figure 3D). All bands were completely inhibited by 1-10 phenanthroline (S3-B).

3.4-Infectiveness *in vivo*

The lesion enlargements in hamsters were significantly highest in L(V)l in the 2nd week of infection (p=0.001) with a lesion size of 0.81±0.23 mm, after that, there was no significant difference in the lesion enlargement compared to the lesion size at the 15th week (p>0.05). From the 5th week until the end, the L(V)b-M2314 had the largest lesion size (p=0.001). At the end of 105 days (15th week) of infection, the mean of lesion enlargements in animals infected with L(V)b-M2314 was 3.49±0.98 mm and in those infected with L(V)l and L(V)n were 0.68±0.25 and 0.08±0.12 mm, respectively (Figure 4A). The lesions of L(V)b-M2314 evolved to ulceration, those of L(V)l showed only swelling and those infected with L(V)n did not show any visible change (Figure 4B).

In terms of parasite load in lymph node by limiting dilution, all species were positive after two weeks of infection (6.58±1.46, 9.35±1.25 and 7.4±1 0.73 log of parasites/g of tissue for L(V)b-M2314, L(V)l and L(V)n, respectively), being the parasite load of animals infected with L(V)b-M2314 the lowest (p=0.007). In the following two parasite loads (8th and 15th weeks postinfection), L(V)l and L(V)n did not show detectable parasite and the load of L(V)b-M2314 had a slight growth to 7.46±1.55 and 8.26±2.00 log of parasites/g of tissue, respectively (Figure 4C). The parasite loads by limiting dilution of liver and spleen were negative for all species in all analyzed times.

However, the parasite loads of spleen by qPCR detected the parasite in infections caused by L(V)b-M2314 at the 8th week postinfection with 13.61±9.39 parasites/g of tissue and it remains positive at the 15th week postinfection with 10.27±1.79 parasites/g of tissue. In the animals infected by L(V)l, the parasite load was detected only in the second week with 2.68±1.71 parasites/g of tissue (Figure 4D). Finally, the animals infected by L(V)n did not have any parasite detectable in all times of spleen parasite load. The animal weights were not affected by *Leishmania* infections (S4).

4-Discussion

In the *in vitro* cultures of the studied strains/species, remarkable differences in promastigotes forms were observed and confirmed by the morphological assay (Figure 1). A study done in 1987 found that L(V)l had mean body length of 17.43±2.91 μm (10.94-22.17 μm) (Silveira et al., 1987)

and another study of 1989 found that L(V)n had body length of 10.11 ± 3.02 μm (4.60-19.93 μm) (Lainson and Shaw, 1989). Comparing to our results (Figure 1B), there are agreement that L(V)1 still has greater body length than L(V)n. Despite the differences in body length when compared to those found in this study, which could be a result of the methodology used in these articles that are not described. The comparison of body length results for L(V)b was not possible, because it was not found in the literature. We believe that the difference observed in body length cannot be discussed in terms of the specie identification, since they are related with promatigote stages and parasite virulence.

Based on the body length, body width and the proportion of flagellum and body is possible to suggest that the majority of promastigotes in L(V)n culture were procyclic promastigotes. While the L(V)l culture is mostly nectomonad promastigotes. Finally, in the three L(V)b cultures the main form was the metacyclic promastigote (Table 1). The promastigotes are flagellar forms present in vertebrate host (vector) and *in vitro* culture, while an aflagellar form called amastigote is present in mammalian host (CDC, 2018). In the vector, the promastigote forms have different evolutionary forms that are from procyclic to metacyclic promastigote, including more three different forms (Gossage et al., 2010; Rogers et al., 2002). According to Gossage (2002), these forms are identified based on the body length, body width, and the proportion of flagellum and body. The infective potential of each form is not clear understood, but there is the knowledge that metacyclic promastigotes are highly infective and that these different forms can also be observed in *in vitro* culture (Gossage et al., 2010; Rogers et al., 2002). In the present study, the different results of promastigote forms (body length, body width and the proportion of flagellum and body) may reflect their differences in infectivity and differences in membrane constitution (as peptidases), which were observed in the zimografic assay of *Leishmania* peptidases (Figure 3A). The forms and peptidases contribute to understand the virulence, infectivity and *in vivo* infection development observed in this study.

The different patterns of *Leishmania* protease activity were observed among the species (Figure 3A). Peptidases are a group of enzymes produced by both parasites and host. They are related with different stages of leishmaniasis and can directly influence the course of the disease. The *Leishmania* peptidases are related to several developmental and proliferation processes, contributing to the interaction with both hosts (vertebrates and invertebrates) and evasion of immune response by the parasites (Mckerrow et al., 1993; Mottram et al., 1998; Vermelho et al., 2007; Yao et al., 2003; Isnard et al., 2012;). Similar to our results, Cuervo et. al (2005 and 2008) compared the peptidase active profile of different strains of L(V)b isolated from patients with different clinical manifestations and found different peptidases partners among them, ranging from 50-125 kDa, being the set of bands expressed in mucosal and cutaneous leishmaniasis distinct. Thus, the peptidases patterns appear to be related to different clinical manifestations, since L(V)b is related with the mucosal and cutaneous leishmaniasis, and the L(V)l and L(V)n are related only with cutaneous form (CDC, 2017). The different infection developments in golden hamster (lesion development and parasite load) caused by these three species investigated (Figure 4) could also be related to the difference in peptidase activity profile. Thus, we encourage studies on peptidases identification to clarify the pathogenicity of each specie.

In the L(V)b strains complex, the patterns of *Leishmania* peptidase activity among L(V)b strains were equal, but the amount of peptidase activity in two bands out of the three expressed were decreased in the L(V)b-M2314 strain, that was isolated from a patient with good response to Glucantime® treatment. Based on the study of Lima et. al (2009), that found a low number of peptidase bands in a nonvirulent strain of L(V)b (1 bands) compared to a virulent one (four bands), it is possible to affirm that this decrease in L(V)b-M2314 peptidases is related with less virulent parasite when compared with the other two strains.

Biochemical characteristics of these peptidases showed that all of them belong to the metalloproteases class since there were abrogated by 1-10 phenanthroline, a specific inhibitor of this class of enzyme (Cuervo et al., 2008). One of the most important metalloprotease presents in the

membrane of *Leinshmania* is the glycoprotein 63 (GP-63) being strong related to the parasite virulence (Oliveira et al., 2013). This is a multifunctional enzyme, that is associated with the inactivation of complement compounds (Isnard et al., 2012), degradation of extracellular matrix contributing to cell migration (Isnard et al., 2012; Yao et al., 2003), inhibition of natural killing cells and activation of tyrosine phosphatase in the host (Gomez et al., 2009; Shio and Olivier, 2010). Resting on these facts, further investigations in GP-63 would give major contributions in the virulence understanding of *Leishmania (Viannia)* spp, highlighting that the role of *Leishmania* metalloproteases remain unclear particularly for *L. (V.) lainsoni* and *L.(V.) naiffi*.

The host metalloprotease also are involved in the leishmaniasis pathogenesis (Campos et al., 2014; De Oliveira et al., 2013; Fraga et al., 2012; Islam et al., 2013; Maretti-Mira et al., 2011, 2010), but are few studies on New World *Leishmania* species (Campos et al., 2014; Fraga et al., 2012; Maretti-Mira et al., 2011, 2010). The host peptidase profiles of macrophages infected or not with *Leishmania (Viannia)* spp. were the same, with six bands belonging to the metalloproteases class based on their biochemical characteristics(Cuervo et al., 2008). According to the molecular weight, bands found in this study could be a MMP complex probably involving MMP-9 (130kDa), pro-MMP-9 (98 KDa), pro-MMP-2 (72 kDa), MMP-2 (68 kDa), and two metalloprotease degraded products (37 and 34 kDa). Lee et al. (2012) found that pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 are expressed constitutively in J774A.1 macrophages, which is in concordance with our results. This technique of zymography with the substrate gelatin allows the detection of gelatinases such as MMP-9 and MMP-2, others metalloproteases such as MMP-13, MMP-8 and MMP-1 could appear with a weak signal since gelatin is not their main substrate. This technique can detect the approximate amount of 10 pg of MMP-2 (Snoek-van Beurden and Von den Hoff, 2005). Most metalloproteases are secreted in form of a precursor called pro-metalloprotease that would be activated by other compounds (other MMPs or serine proteases) and became the metalloprotease itself (Klein and Bischoff, 2011; Nagase et al., 2006). In addition, they can make complex with other substances that is not dissociated in zymographic assay such as MMP-9 and a 25-kDa protein (microglobulin), giving

a band at 125 kDa and a dimer at 215 kDa. This technique can also detect the degradation products of these metalloproteases that would have low molecular weight (Snoek-van Beurden and Von den Hoff, 2005).

Nowadays the MMP-9 and MMP-2 have been related to the immunopathogenesis of leishmaniasis, being the high expression of MMP-9 related with clinical manifestations of post kalazar dermal leishmaniasis (Islam et al., 2013) and the dissemination of parasites in the mucosal form of diseases (Maretti-Mira et al., 2011). Both metalloproteases were related to therapeutic failure (Maretti-Mira et al., 2010) and progression of tegumentary leishmaniasis (Campos et al., 2014). In general, the MMP-9 and MMP-2 are associated with severe and benign form of diseases, respectively. These findings are in disagreement with ours, because we found out that the low expression of the band 72 kDa, supposedly pro-MMP-2, was diminished in the macrophages infected with L(V)n, the strain that presented to be less virulent in animal model. One explanation for this is that other cell lines, despite the macrophages, can produce metalloproteases in leishmaniasis, such as monocyte lines (Campos et al., 2014) and activated neutrophils (Wéra et al., 2016) and all the mentioned studies were made in patient's blood or biopsies, a much more complex physiological system than just macrophages. Another important point is that in literature was found only few studies that investigate these metalloprotease in cutaneous and mucosal leishmaniasis (Campos et al., 2014; Fraga et al., 2012; Maretti-Mira et al., 2011, 2010), suggesting that these results are preliminary and need more confirmations.

In addition to the promastigote and peptidase assays, we investigated the amastigote infection on macrophages. The absence of difference in the infection indexes at 0 h among the strains of L(V)b, L(V)l and L(V)n may reflect that the macrophage-*Leishmania* interaction mediated by ligand-receptor are not so different among them. However, there is evidence that molecules of parasites such as GP-63 and lipophosphoglycan involved in this and others virulence processes are redundant expressed in *Leishmania* sp.(Brittingham et al., 2001; Yao et al., 2003). Then, they could operate in a compensatory manner depending on the abundance of them (Matta et al., 2010), being a limitation

of the used method. The infection indexes founded in our study showed that L(V)l was less infective to macrophages than the others and, interestingly, it was the only tested specie which the infected index at 0 h was significantly decreased 24 h later (Figure 2). This result showed that the macrophages are able to control better the infection caused by L(V)l, as also observed in animal model.

Considering the importance of nitric oxide for parasites elimination (Maksouri et al., 2017), the nitrite-derived production was investigated. In the present study, no production of nitrite-derived by macrophages infected with *Leishmania (Viannia)* spp. was detected. Matta et al. (2010) compared the L(V)n with L(V)b and *L. (V.) guyanensis in vitro* and did not report any difference in their infection indexes, similar to our results. In relation to the findings involving the strains of L(V)b, no significant difference was found neither in the infection indexes nor in the nitrite-derived production, which are similar to the results of Fernandes et. al (2016). However, Campos et. al (2008) observed that L(V)l and L(V)n are higher producers of nitrite-derived in relation to a L(V)b isolated from a CL without statistical difference. These results are in discordance with our work and this could be explained by the fact that the strains used by Campos et. al (2008) are different from ours and there is intraspecific genetic polymorphism already reported by L(V)b (Cupolillo et al., 2003; De Oliveira et al., 2004) that may be extended to other *Leishmania* species. Interesting, when these strains of L(V)b, L(V)n and L(V)l were compared by Campos et. al (2008) in terms of infection indexes, no difference was found corroborating our results.

An experimental study carried out with five strains of L(V)b, including the L(V)b-M2314 and L(V)b-M3476 did not shown any significant difference in terms of lesion enlargement, weight and parasite load (Fernandes, 2015). These finds associated with our results *in vitro*, that did not show any significant difference among the strains of L(V)b, guided our decision of procced with the *in vivo* experiment using only one strain representative of the L(V)b species, that was L(V)b-M2314.

As far as we are aware, it is the first study that compares L(V)b-M2314, L(V)l and L(V)n in an experiment *in vivo* (Figure 4). In our experiment, the highest virulence of L(V)b-M2314 was evident because it presented the worse lesion progression and parasite dissemination to lymph node

and spleen. These results of L(V)b-M2314 were expected, since inoculum of L(V)b higher than $1x10^6$ in golden hamster results in ulcerative lesions and parasite dissemination (Ribeiro-Romão et al., 2014). The same behavior found *in vitro* (Figure 2) for L(V)l was also observed *in vivo.* Initially, the L(V)l presented to be the most infectivity specie when compared to the other two, with a sharp increase in lesion growth, and positive lymph node and spleen loads followed by a stabilization and remission, respectively, reinforcing the assumption that the host immune system controls the infection caused by L(V)l in a special manner. Further studies that investigate other mechanisms which explains this behaviour of L(V)l *in vitro* and *in vivo* would be worth for a better understanding of immune mechanisms in leishmaniasis that could instigate new researches, such as cytokines and metalloproteases involved in the leishmaniasis pathogenesis.

The infection caused by L(V)n in golden hamster showed an interesting behavior. The resistance of animal model to L(V)n infection or absence of virulence by the parasite associated to *in vitro* infectivity was also observed by Lainson and Shaw (1989) and Campos et al (2008). In humans, L(V)n is able to develop cutaneous leishmaniasis (WHO, 2010; Figueira et al., 2014). In 2015, a study in surrounding areas of Manaus-Brazil attributed 27% (8/30) of CL cases to the specie *naiffi* (Fagundes-Silva et al., 2015). Differently from previous findings that related L(V)n with self-limited infection and good response to the treatment (Naiff et al., 1991; Pratlong et al., 2002; Van Der Snoek et al., 2009), this study did not report any self-limiting nature in L(V)n infection and observed poor response (2/8) to pentamidine or Glucantime® treatment (Fagundes-Silva et al., 2015). In regard to these facts, L(V)n is capable of developing leishmaniasis in humans but not in hamsters. Studies that search for animal model able to reproduce the human infection is of critical importance in face of the spreading in cases of leishmaniasis caused by this specie (Pratlong et al., 2002; Figueira et al., 2008; Fagundes-Silva et al., 2015).

The results reported in this study did not find any remarkable difference among the strains of L(V)b, but they suggest that L(V)b, L(V)l and L(V)n have difference in virulence and peptidase activity profile (Table 1) that could be related to their clinical manifestation in humans. According to

these findings, the most virulent specie is L(V)b and it is the only one related with cutaneous and mucosal leishmaniasis. Besides, it also supports the fact that L(V)b is the major responsible for CL in the New World and that the other two species are not so frequent in human infection, not just because of their vectors are low anthropophilic, but also because they are less virulent.

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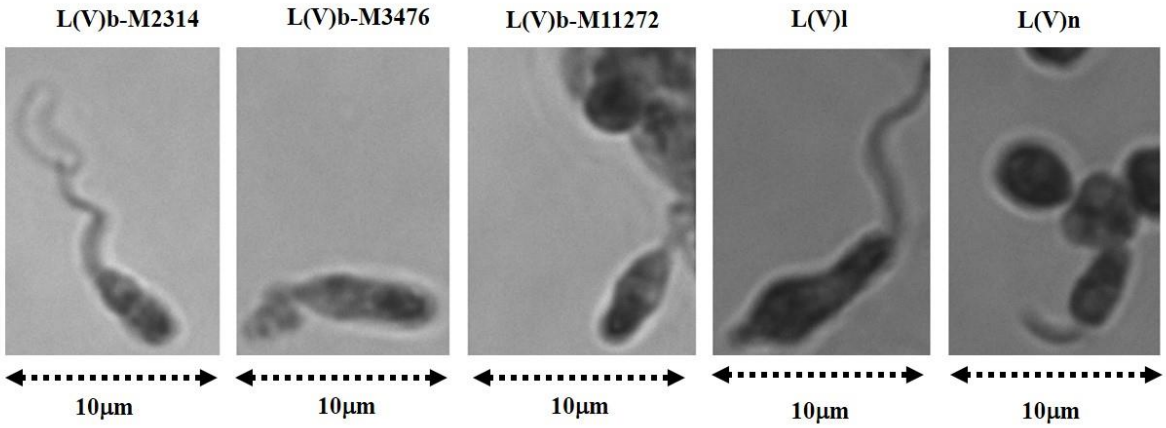
FIGURES AND LEGENDS

A



B

Leishmania (V.) spp.	Form	Size (µm)± SD (µm)
L(V)b-M2314	Fusiform	8.32± 1.64^{ab}
L(V)b-M3476	Fusiform	8.50± 1.33^{ab}
L(V)b-M11272	Fusiform	7.55± 1.27^{ab}
L(V)l	Fusiform	11.71± 1.95^{a}
L(V)n	Oval	4.82± 0.62^{b}

Figure 1: Morphologic assay of *Leishmania (Viannia)* spp. A- Promastigote forms stained by Panotico kit observed in EVOS FL Imaging System at 100x magnification. B- Board with body length and promastigote forms of *Leishmania (Viannia)* spp. The mean and standard deviation (SD) was calculated based on the measure of 2 promastigotes per field of at least 10 fields in the EVOS FL Imaging System. L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)b-M3476: *L. (V.) braziliensis* (MHOM/BR/2009/3476); L(V)b-M11272: *L. (V.) braziliensis* (MHOM/BR/1987/M11272); L(V)l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424)*;* L(V)n: *L. (V.) naiffi* (MDAS/BR/79/M5533). p=0.001 by ANOVA and Tukey post hoc tests.

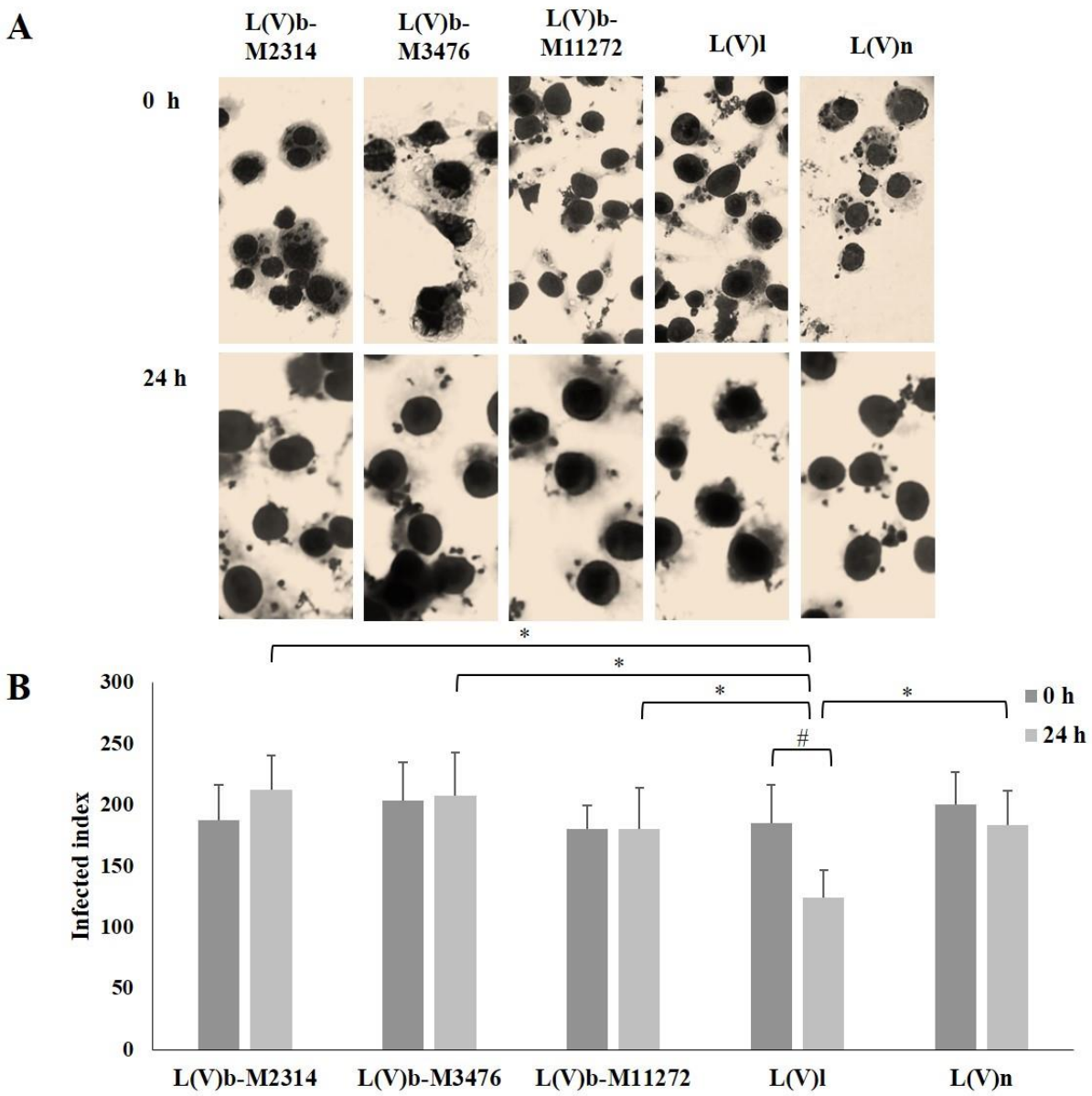


Figure 2: Infectiveness *in vitro*. A- Macrophages infected with *Leishmania (Viannia)* spp. at time zero (0 h) and macrophages infected with *Leishmania (Viannia)* spp. after 24 hours of infection (24 h) observed in microscope at 100x magnification. C - Infection indexes at 0 h and after 24 h of infection. L(V)b-M11272: *L. (V.) braziliensis* (MHOM/BR/1987/M11272); L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)b-M3476: *L. (V.) braziliensis* (MHOM/BR/2009/3476); L(V)l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424)*;* L(V)n: *L. (V.) naiffi* (MDAS/BR/79/M5533). * p =0.001 by Kruskal-Wallis and Mann-Whitney pairwise tests. # p =0.001 by Mann-Whitney test. The results are expressed in mean and standard deviation of three independent experiment.

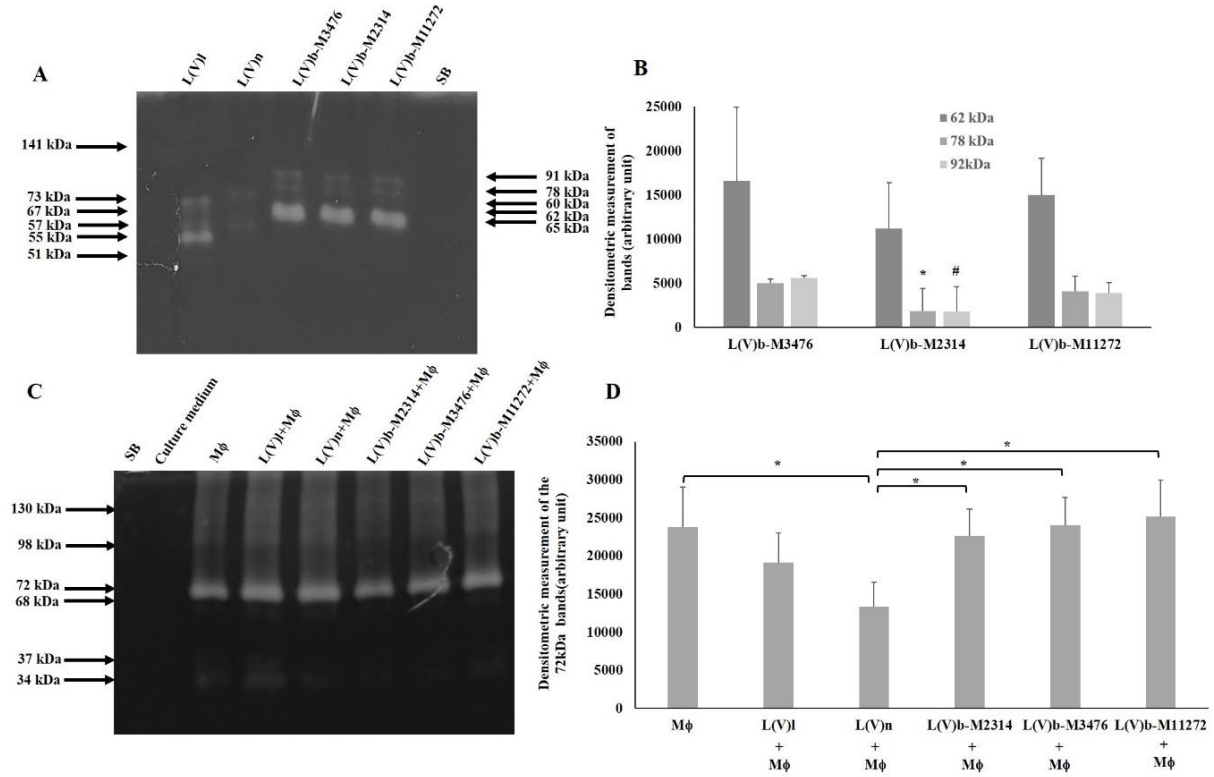
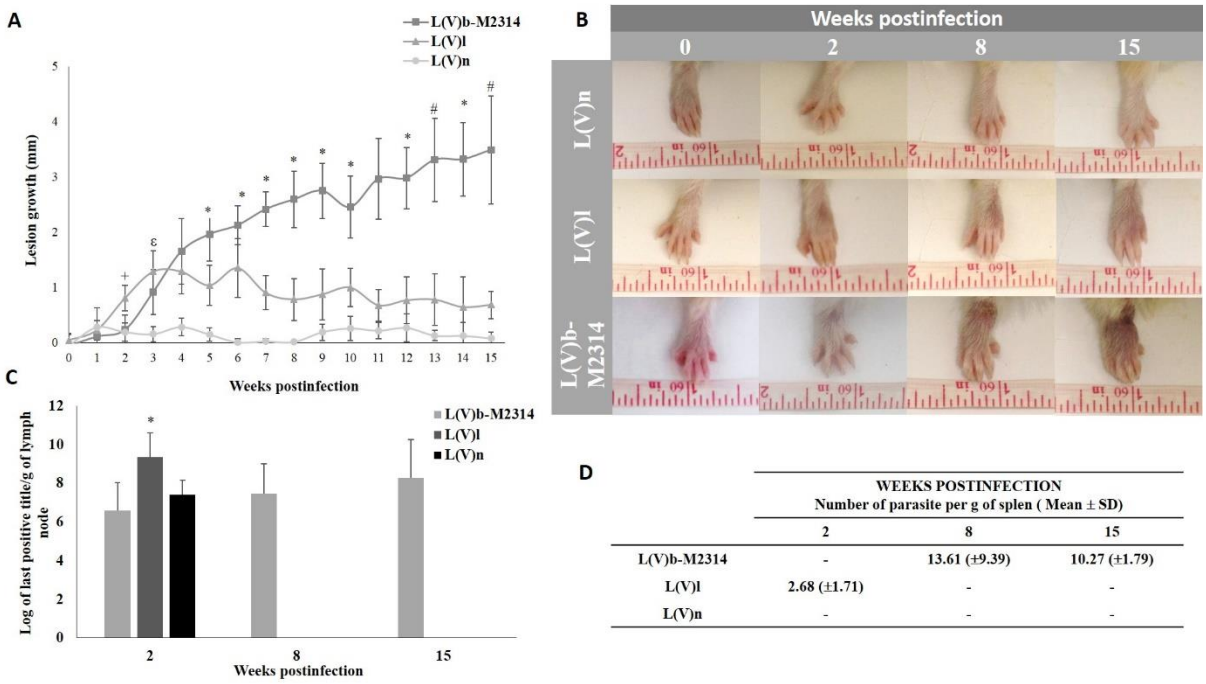


Figure 3- Peptidase proteolytic profile. A- Peptidase proteolytic profile of enzymes present in the membrane of promastigotes of *Leishmania (Viannia)* spp. in SDS-page. The *Leishmania (Viannia) lainsoni* (MHOM/BR/8l/M6424) [L(V)l] expressed seven bands (141, 73, 67, 60, 57, 54 and 51 kDa); the *Leishmania (Viannia) naiffi* (MDAS/BR/79/M5533) [L(V)n] expressed four bands (78, 62, 55 and 57 kDa); and the strains *L. (V.) braziliensis* MHOM/BR/1987/M11272 [L(V)b-M11272], *L. (V.) braziliensis* MHOM/2003/2314 [L(V)b-M2314] and *L. (V.) braziliensis* MHOM/BR/2009/3476 [L(V)b-M3476] expressed three bands (92, 78 and 62 KDa). B- Densitometric measurement (arbitrary unit) of bands expressed in the strains of *Leishmania (Viannia) braziliensis*; * p= 0.004 and # p= 0.003 by Kruskal-Wallis and Mann-Whitney pairwise tests. C- Peptidase proteolytic profiles of enzymes secreted by macrophages (Mϕ) alone and infected by *Leishmania (Viannia)* spp.; all sample presented the same peptidase proteolytic profile with six peptidases: 130, 98, 72, 68, 37 and 34 kDa. D- Densitometric measurement (arbitrary unit) of the band of 72 kDa expressed in the zymographic assay of secreted enzymes by macrophages in presence and absence of *Leishmania (Viannia)* spp; * p= 0.005 by Kruskal-Wallis and Mann-Whitney pairwise tests. Sample buffer (SB) is an internal control. The results are expressed in mean and standard deviation of two independent experiment.



D

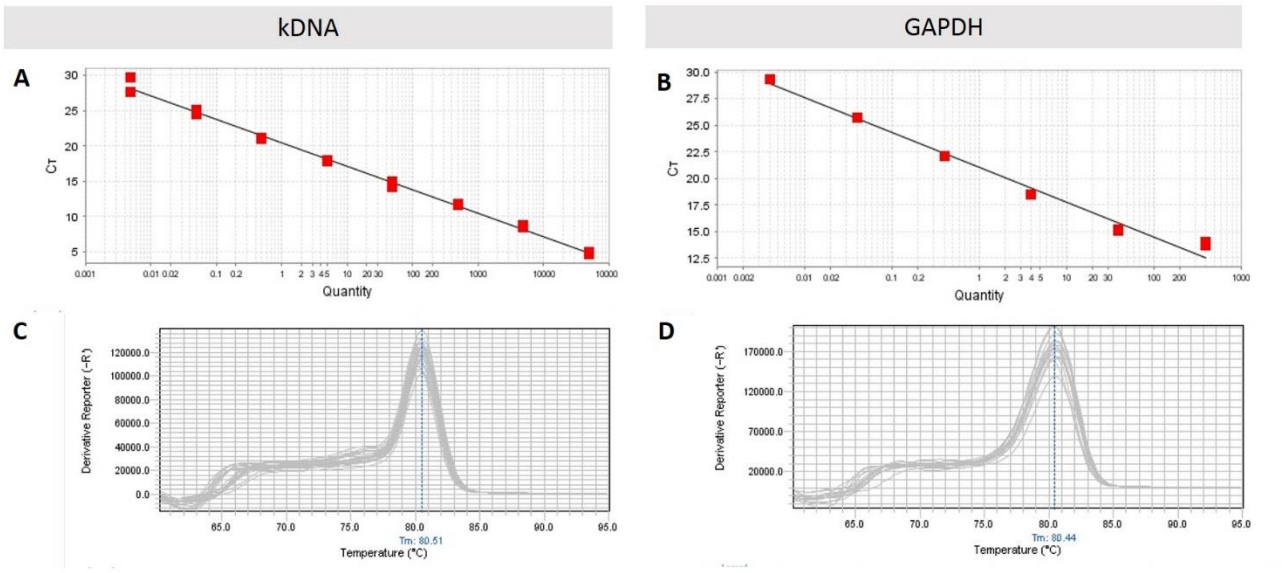
	WEEKS POSTINFECTION Number of parasite per g of splen (Mean ± SD)		
	2	8	15
L(V)b-M2314	-	13.61 (±9.39)	10.27 (±1.79)
L(V)l	2.68 (±1.71)	-	-
L(V)n	-	-	-

Figure 4: Infectiveness *in vivo*. A- Lesion size throughout the weeks of infection; + there were significant differences among the species, being the lesion sizes caused by *L. (V.) lainsoni* [L(V)l] the biggest when compared to the lesion size of infections caused by the other two species, which were statistically equal (p=0.001); * all species had significant differences in lesion sizes, being the lesion sizes caused by *L. (V.) braziliensis* (MHOM/2003/2314) [L(V)b-M2314] the biggest (p=0.001); ε the lesion sizes caused by *L. (V.) naiffi* [L(V)n] was the smallest one and there was no difference in lesion sizes between the others (p=0.001); # the lesion sizes in animals infected by L(V)b-M2314 was the largest when compares to the others that were statistically equal (p=0.001). The statistical tests used were Kruskal-Wallis and Mann-Whitney pairwise. B- Infected right hind paws at the 0, 2nd, 8th and 15th week after infection. C- Bar graph of parasite loads on right popliteal lymph nodes by limiting dilution; *there was significant difference among the parasite loads (p=0.007) by Kruskal-Wallis and Mann-Whitney pairwise tests. D- Board with the parasite loads on spleen by real-time PCR. The results are expressed in mean and standard deviation.

Table 1- Main differences among studied *Leishmania (Viannia)* spp.

Leishmania (Viannia) spp.	Predominant promastigote form in culture	Metalloproteases profile presents in the membrane of promastigotes (kDa)	Lesion enlargment at 15th week of infection Mean ±SD (mm)	Parasite load on lymph node at 2nd week of infection Mean ±SD [log(last positive title/ g of tisssue)]	Parasite load on lymph node at 8th week of infection Mean ±SD [log(last positive title/ g of tisssue)]	Parasite load on lymph node at 15th week of infection Mean ±SD [log(last positive title/ g of tisssue)]
L. (V.) naiffi MDAS/BR/79/M5533	Procyclic	78, 62, 55 and 57	0.08±0.12	7.4±1 0.73	Negative	Negative
L. (V.) lainsoni MHOM/BR/8l/M6424	Nectomonad	141, 73, 67, 60, 57, 54 and 51	0.68±0.25	9.35±1.25	Negative	Negative
L. (V.) braziliensis MHOM/2003/2314	Metacylicl	92, 78 and 62	3.49±0.98	6.58±1.46	7.46±1.55	8.26±2.00
L. (V.) braziliensis MHOM/BR/2009/3476	Metacylicl	92, 78 and 62	–	–	–	–
L. (V.) braziliensis MHOM/BR/1987/M11272	Metacylicl	92, 78 and 62	–	–	–	–

SD- standard deviation



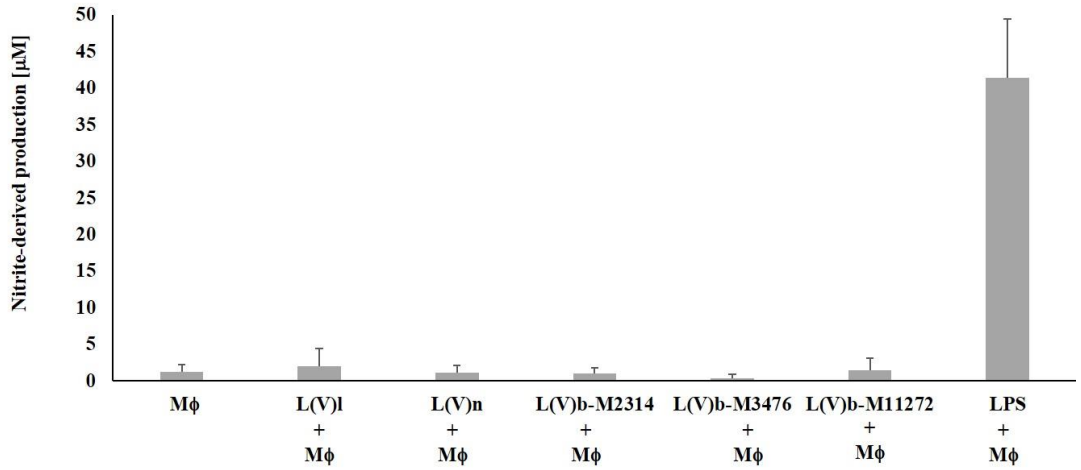
E

Efficiency	99.951
Linear Coefficient(r^2)	0.996

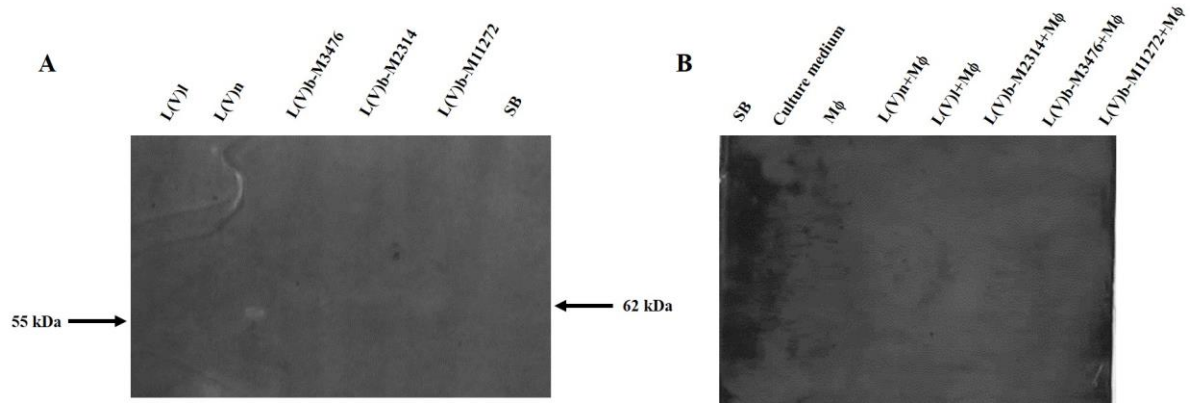
F

Efficiency	101.599
Linear Coefficient(r^2)	0.987

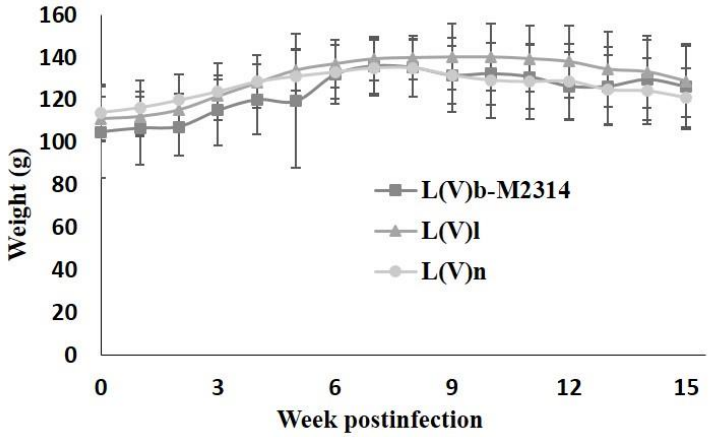
Supplementary data 1: Standardization of qPCR for parasite load in the spleen of golden hamsters. A- Standard curves for golden hamster GAPDH. B- Standard curves for parasite kDNA. C- The melting curve of golden hamster GAPDH. D- The melting curve of parasite kDNA. E- PCR efficiency and linearity coefficient of the kDNA target. F- PCR efficiency and linearity coefficient of the GAPDH target.



Supplementary data 2: Nitrite-derived production. Production of nitrite-derived in µM by J774A.1 macrophages alone and in response to *Leishmania (Viannia)* spp. infection. Mϕ: J774A.1 macrophages; L(V)l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424)*;* L(V)n: *L. (V.) naiffi* (MDAS/BR/79/M5533); L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)b-M3476: *L. (V.) braziliensis* (MHOM/BR/2009/3476); L(V)b-M11272; *L. (V.) braziliensis* (MHOM/BR/1987/M11272); LPS: lipopolysaccharide (positive control of nitrite-derived production). The results are expressed in mean and standard deviation of three independent experiment.



Supplementary data 3: Inhibition peptidase proteolytic assay. A- Peptidase proteolytic profile of enzymes present in the membrane of promastigotes of *Leishmania (Viannia)* spp. in SDS-page treated with 10 mM of 1-10 phenanthroline. The *L. (V.) lainsoni* (MHOM/BR/8l/M6424) [L(V)l] had the activity of six peptidase bands completely inhibited (141, 73, 67, 60, 57 and 51 kDa) and the peptidase band of the 55 kDa was partially inhibited; the *L. (V.) naiffi* (MDAS/BR/79/M5533) [L(V)n] hab all peptidase bands completely inhibited (78, 62, 55, 57 kDa); and the strains of *L. (V.) braziliensis* MHOM/BR/1987/M11272 [L(V)b-M11272], *L. (V.) braziliensis* MHOM/2003/2314 [L(V)b-M2314] and *L. (V.) braziliensis* MHOM/BR/2009/3476 [L(V)b-M3476] hab two peptidases bands inhibited (92 and 78 kDa) and the peptidase band of 62 kDa was partially inhibited. C- Peptidase proteolytic profiles of enzymes secreted by macrophages (Mϕ) alone and infected by *Leishmania (Viannia)* spp.; all strains had their peptidases band inhibited by 5 mM of 1-10 phenanthroline; Sample buffer (SB) is an internal control.

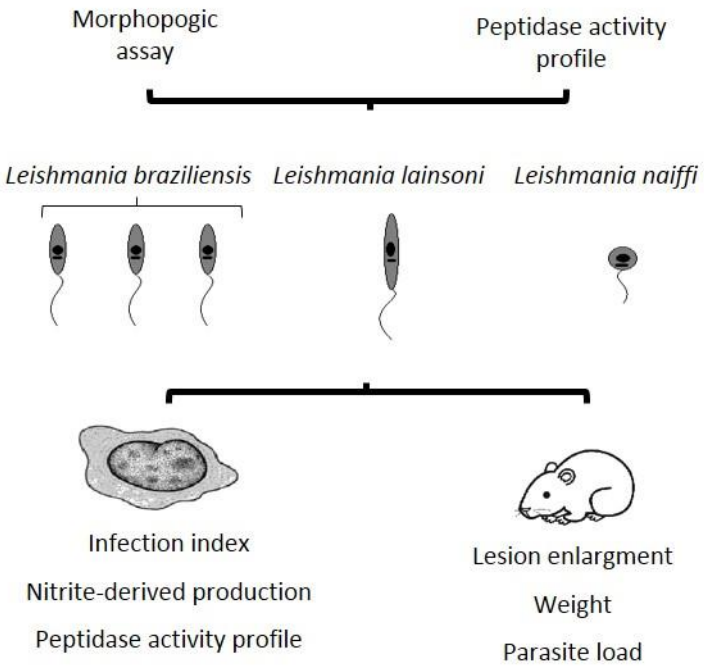


Supplementary data 4: Weight of animals throughout the weeks of infection. L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424)*;* L(V)n: *L. (V.) naiffi* (MDAS/BR/79/M5533). The results are expressed in mean and standard deviation.

HIGHLIGHTS

- *Leishmania braziliensis, L. naiffi* and *L. lainsoni* had different peptidase profile.
- *L. lainsoni* had the lowest infection index in macrophages.
- *L. braziliensis* is most virulent specie *in vivo.*
- The different among the three species could explain their leishmaniasis outcomes.

GRAPHICAL ABSTRACT



CAPÍTULO III

CONCLUSÕES

Os resultados relatados neste artigo sugerem que L(V)b, L(V)l e L(V)n possuem diferenças nos perfis enzimáticos e virulência que podem estar relacionadas às suas manifestações clínicas em seres humanos. De acordo com esses achados, a espécie mais virulenta é a L(V)b e na literatura, é a única relacionada com a leishmaniose cutânea e mucosa. Além disso, também fornece evidências que corroboram o fato da L(V)b ser a principal responsável pela leishmaniose cutânea no Novo Mundo. As outras duas espécies não são tão frequentes na infecção humana, não apenas por seus vetores serem antropófilos baixos, mas também porque são menos virulentas.

PERSPECTIVAS FUTURAS

- Identificar as metaloproteases que foram diferentemente expressas entre as espécies.
- Avaliar a expressão das metaloproteases *in vitro* em células polimorfonucleares presente no sangue periférico de humanos
- Avaliar os aspectos histológicos das infecções *in vivo* decorrentes de cada espécie.
- Avaliar diferenças nos parâmetros imunológicos do hospedeiro em resposta a infecção por estas três espécies.